Biochimie 99 (2014) 71–76

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

The cell cycle arrest and the anti-invasive effects of nitrogen-containing bisphosphonates are not mediated by DBF4 in breast cancer cells

Mahdieh Mansouri [a], Seyed Abbas Mirzaei [a,b], Hermann Lage [b], Seyyedeh Soghra Mousavi [a], Fatemeh Elahian [a,b,*]

[a] Department of Pharmaceutical Biotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Iran
[b] Institute of Pathology, Charité Campus Mitte, University Medicine Berlin, Berlin, Germany

ARTICLE INFO

Article history:
Received 19 July 2013
Accepted 11 November 2013
Available online 26 November 2013

Keywords:
Bisphosphonates
Cell cycle
DBF4
Invasion assay
Risedronate
Zoledronate

ABSTRACT

Recent work has shown that a DBF4 analog in yeast may be a target of nitrogen-containing bisphosphonates. DBF4 is an essential protein kinase required for DNA replication from primary eukaryotes to humans and appears to play a critical role in the S-phase checkpoint. It is also required for cell migration and cell surface adhesion. The effects of Pamidronate, risedronate, or zoledronate on cell viability and DBF4 expression were measured via MTT assays and western blotting. In addition, FACS cell cycle analyses and invasion assays were conducted in cells in the presence of nitrogen-containing bisphosphonates to identify any correlations between DBF4 expression and S-phase arrest or anti-invasive effects of the bisphosphonates. Zoledronate transiently down-regulated DBF4 expression in all three cell lines in the first 24 h of the experiment, but after 72 h, DBF4 expression returned to the control levels in all treated cells. Following treatment of the tumor cells with the bisphosphonates, the number of cells in S-phase was increased. Pamidronate and zoledronate showed anti-invasive effects in BT20 cells. The anti-invasive effects of pamidronate, risedronate and zoledronate appeared after 48 h of exposure. In MDA-MB231 cells a reduction of invasiveness was only observed after 72 h of the pamidronate exposure. We finally concluded that the anti-invasive and cell cycle arrest-inducing effects of nitrogen-containing bisphosphonates are not DBF4 mediated, and other mediators are therefore needed to explain the observed complex behaviors.

1. Introduction

Bisphosphonates are a series of synthetic compounds that are widely used in the treatment of bone metabolic diseases associated with high bone resorption, including destructive arthropathy, fibrous dysplasia, heterotopic ossification, osteogenesis imperfecta, and Paget's disease [1]. Bisphosphonates are chemically and enzymatically stable and resemble pyrophosphate analogs characterized by a P–C–P bond [2]. The germinal carbon in this moiety is covalently bonded to two different lateral chains that are usually referred to as the R1 and R2 groups. R1 and R2 confer a pronounced binding affinity for hydroxyapatite and antiresorptive properties in bone, respectively. Based on their molecular structure and mechanism of action, two distinct types of bisphosphonates have been defined. Non-nitrogen-containing bisphosphonates (non-NBPs), such as clodronate and etidronate, are metabolized to form potentially cytotoxic analogs of ATP that accumulate in cells and deactivate osteoclasts, leading to cell death [1]. In contrast, nitrogen-containing bisphosphonates (NBPs), such as alendronate, ibandronate, pamidronate, risedronate, and zoledronate, inhibit intracellular farnesyl diphosphate synthase (FPPS) in the mevalonate pathway, which results in decreased levels of farnesyl diphosphate and geranylgeranyl diphosphate and inhibition of the prenylation of GTP-binding proteins (such as Ras, Rho, and Rac), which is considered to represent the main mechanism of osteoclast activity inhibition and apoptosis promotion [3,4].

Although these bioactive agents are only FDA approved for the treatment of bone metabolic diseases, they have recently gained attention due to their strong apoptosis induction effect in human myeloma cells [5], immunomodulation and stimulation of cytotoxic T-cells targeting tumor cells [4], inhibition of tumor angiogenesis [3], inhibition of the adhesion of invasive cancer cells to bone matrices [6], inhibition of cell invasion and proliferation, and

* Corresponding author. School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran. Tel.: +98 241 4273636; fax: +98 241 4273639.
E-mail addresses: elahian@zums.ac.ir, dr.elahian@yahoo.com (F. Elahian).

0300-9084/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biochi.2013.11.010

promotion of cell cycle arrest. Significant progress has been made in uncovering the mechanisms underlying these anticancerous and immunomodulatory properties. Induction of caspase activity (especially caspase-3 or caspase-7), progressive decreases in the bcl2/bax ratio, and inhibition of the mevalonate pathway are important apoptotic mechanisms associated with the effects of bisphosphonates on tumor cells [4,5]. Interestingly, NBPs down-regulate vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) expression and show potential anti-angiogenetic effects [7]. However, little is known about the molecular mechanisms underlying the anti-invasive and cell cycle arrest-inducing effects of NBPs, and it appears that more complex pathways may be involved. A large-scale experiment examining different yeast mutants in the presence of sub-lethal doses of some NBPs revealed the YDR052C protein (yeasts analog of human DBF4) as a secondary cellular target for these agents [8]. DBF4 is a regulatory subunit of the Cdc7 serine/threonine-specific protein kinase, which is a checkpoint effector enzyme for the initiation of DNA replication and entry into S-phase of the cell cycle [9]. Cdc45 and MCM proteins that bind to replication origins and activate the replication initiation complex are phosphorylated by Cdc7-DBF4. Cdc7 is consistently expressed during the cell cycle, while DBF4 decreases during G1 phase and increases during S and G2 phases. Therefore, up-regulation of DBF4 promotes S-phase [10]. It has also been demonstrated that DBF4 phosphorylates integrin and that depletion of DBF4 kinase reduces cell attachment to collagen-I-coated surfaces in some cells. DBF4 also plays a critical role in cell adhesion and migration, possibly through regulation of actin cytoskeleton arrangement [11]. Although yeast experiments have shown that the anti-invasive and S-phase arrest-inducing effects of NBPs may be mediated via the YDR052C protein, further studies are needed to confirm these mechanisms in human cells. Considering all of these findings, the present study focuses on analysis of the relationship between NBP treatment and DBF4 expression levels in three different human cell lines and the potential consequences of the biological effects of NBPs on the cell cycle and migration behavior.

2. Materials and methods

2.1. Chemicals and media

RPMI-1640 medium, trypsin, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). Pamidronate, risedronate, zoledronate, monoclonal anti-human β-actin (mouse IgG1), monoclonal anti-human DBF4 (goat IgG) antibodies, and an HRP-conjugated secondary antibody (goat anti-mouse IgG) were obtained from Santa Cruz biotechnology (California, USA). Complete Mini Protease Inhibitor Cocktail Tablets™ came from Roche Applied Science (Mannheim, Germany). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Cell lines and cell proliferation assay

The BT20, MDA-MB231, and T47D human epithelial breast cancer cell lines were purchased from the Pasteur Cell Bank, Iran. The cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at a constant temperature of 37 °C in a humidified CO_2 incubator. Proliferation characteristics were determined according to methods used in previous studies by our group. Briefly, 1000 cells were seeded into each well of 96-well plates in 200 μl of growth medium. The culture medium was refreshed every 2 days. Cell growth was assessed during a 7-day incubation period using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 3 h of incubation with MTT (5 mg/ml), the production of formazan crystals was measured using the TECAN microplate reader at 650 nm. All experiments were run three independent times in triplicate, and the results were expressed as the mean values ± SE [12,13].

2.3. Cell cytotoxicity assay

The cells were cultured at a density of 1000 cells per well in 96-well plates and incubated at 37 °C for 24 h. A stock solution of 10 mM pamidronate, risedronate, and zoledronate in 1× phosphate buffered saline (PBS) was prepared and stored in aliquots at −20 °C. The stock was freshly diluted with culture medium before use and added to microplates at defined concentrations from 0 to 100 μM. Cell viability was evaluated after a 5-day incubation of the treated cells using MTT assays. The IC_{50} was determined as the drug concentration that reduced the surviving fraction of cells in each well by 50% compared to the control. All experiments were performed three independent times in triplicate, and the results were expressed as the mean values ± SE [12,14].

2.4. Protein extraction and quantification

The cancer cell lines were cultured in 6-well plates at a density of 6×10^5 cells/well and incubated at 37 °C for 24 h. The cells were then treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h, followed by being washed twice with cold PBS. Next, 300 μl of RIPA lysis buffer (5 mM EDTA, 150 mM NaCl, 1% v/v Triton X-100, 0.1% w/v SDS, 50 mM Tris–HCl pH 8.0 supplemented with a Complete Mini Protease Inhibitor Cocktail Tablet™) was added to approximately 10^6 exponentially growing cells, followed by incubation on ice for 30 min. Cellular lysates were scraped and collected from culture plates, after which they were centrifuged at 14000× *g* for 10 min, and the supernatant (containing total intracellular proteins) was isolated. The supernatant protein content was measured with the BCA Protein Assay Kit® (Thermo Scientific, Rockford, IL, USA), according the manufacturer's protocol, at 562 nm using serial dilutions of human serum albumin as the standard. Unless otherwise specified, all protein extraction procedures were carried out at 4 °C. Finally, the protein samples were stored at −80 °C until further use [15].

2.5. SDS-PAGE and western blotting analysis

The equivalent of 50 μg of each protein sample was mixed with 6× loading buffer (300 mM DTT, 20% v/v glycerol, 4% w/v SDS, 0.3% w/v bromophenol blue, and 120 mM Tris–HCl pH 6.8), heated for 2 min at 95 °C, and then centrifuged at 12000× *g* for 1 min at 4 °C. The samples were next loaded into the wells of a 12% SDS-PAGE gel and electrophoresed in running buffer (190 mM glycine, 0.1% w/v SDS, 25 mM Tris–HCl pH 8.0) for 1.5 h (90 V). The separated proteins were electro-blotted onto a 0.45 μm nitrocellulose membrane (Portran®, Whatman, Germany), stained with Ponceau-S, and washed twice with TBS-T (TBS containing 0.5% v/v tween-20). The nitrocellulose membranes were pre-blocked with TBS-T containing 5% skimmed milk powder for 1 h at room temperature to reduce nonspecific primary antibody binding. Following three washes with TBS–T, the blots were incubated with a diluted mouse monoclonal antibody directed against either human DBF4 (1:600) or human β-actin (1:600) in TBS-T supplemented with 5% skimmed milk overnight at 4 °C. Then, the membranes were treated with the corresponding HRP-conjugated goat anti-mouse IgG (1:5000) in the same supplemented buffer for 1 h at room temperature. The protein-antibody complexes were visualized using a luminol-based

detection system (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol. Antibody binding was subsequently visualized via exposure of the membrane to blue light-sensitive X-ray film (Kodak, Tokyo, Japan). Immunofluorescence reactions were carried out using at least three independent protein extracts [16].

2.6. Cell cycle analysis

The breast cancer cell lines were seeded into 6-well plates at a density of 6×10^5 cells/well for 24 h. Then, the cells were treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h. Approximately 2.5×10^5 cells were harvested, washed twice using ice-cold PBS, and resuspended in 200 μl of cold PBS. For rapid cell fixation and dispersion, the cell suspensions were added dropwise to 1 ml of cold 70% ethanol and incubated on ice for 45 min. After centrifugation, the cells were resuspended in 1 ml of PI master mix (PBS containing 100 μg/ml RNase A and 40 μg/ml propidium iodide) and incubated in the dark at 37 °C for 30 min. The various cell cycle phases were monitored using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). Cells were excited at 488 nm with an argon laser, and the emission from ten thousand cells was recorded using a 580 nm band-pass filter (FL2-H). For discrimination of G1 doublet cells from G2/M single cells, a dot blot graph of the FL2-W versus FL2-A data was also plotted. The obtained cell cycle profiles were analyzed using CellQuest version 3.2 and WinMDI version 2.8 softwares. All experiments were performed three independent times in duplicate [17].

2.7. Invasion assay

The breast cancer cell lines were seeded into 6-well plates at a density of 6×10^5 cells/well for 24 h, after which they were treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h. Approximately 2×10^5 cells were then harvested in serum-free medium and overlaid in the upper chambers of insertion plates (8 μm pore size; SPL, South Korea) that were uniformly coated with 1 mg/ml BD BioCoat Matrigel™ matrix (BD Biosciences, California, USA). The cells were subjected to a 24 h serum starvation period prior to seeding. The lower chambers were filled with 10% (v/v) FBS-supplemented RPMI-1640 as a chemoattractant. After 24 h of incubation at 37 °C in the humidified CO_2 incubator, the non-invading cells located in the upper portion of chamber were removed with cotton swabs. The numbers of adherent cells on the bottom of each insert were determined by MTT assays using a standard curve prepared from serial dilutions of the corresponding cell line [18].

2.8. Statistical analysis

All experiments were carried out in triplicate at least three independent times, and the means ± standard errors were reported. Quantitative data from the experiments were analyzed using Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Cell proliferation and cell cytotoxicity assays

The cells grew as a monolayer attached to the bottom of the flask and had the appearance of small round clumps. The growth characteristics of the cell lines were determined via MTT dye reduction assays over seven days (Supplementary Fig. 1S). To investigate the effects of NBPs on cell survival, the breast cancer cell lines BT20, MDA-MB231, and T47D were treated with increasing doses of each NBP. A dose–response curve was fitted to our data, and the IC_{50} values were calculated after 5 days of exposure through MTT cell viability assays (Fig. 1). The most cytotoxic compound in the MDA-MB-231 and T47D cells was zoledronate ($P < 0.01$, $P < 0.001$), with IC_{50} values of approximately 7 and 22 μM being recorded, respectively. However, pamidronate was more potent (IC_{50} 7 μM) in the BT20 cell line compared with the other NBPs ($P < 0.01$, Table 1). The obtained data showed that the cytotoxic effects of the NBPs are cell specific.

3.2. Effects of NBP on DBF4 expression

After the extraction of total proteins from the cells, the levels of DBF4 protein expression were determined via western blotting analysis. DBF4 and β-actin appeared in their full-length forms as bands at approximately 37 and 49 kDa, respectively. Although DBF4 expression was not altered in the presence of pamidronate and risedronate in any of the cell lines, zoledronate transiently down-regulated DBF4 expression in all three cell lines in the first 24 h of the experiment. After 72 h, DBF4 expression returned to the control levels in all cells. In fact, there were no differences between the DBF4 levels in any of the NBP-treated cells and control samples after 72 h of treatment (Fig. 2).

3.3. Effects of NBPs on the cell cycle

Cell cycle progression was analyzed via flow cytometry after propidium iodide staining. The distribution of cells in different phases confirmed previous reports that have shown that NBPs can cause cell cycle arrest in S-phase. Following treatment of the tumor cells with 100 μM pamidronate, risedronate, or zoledronate, an increase in the number of cells in S-phase accompanied by a reduction in the proportion in G0/G1 or G2/M phase was noted (Fig. 3 and Supplementary Table 1S). The percentage of cells in S-phase increased progressively in a time-dependent manner with continuing exposure to the three drugs. The increase in the proportion of cells in S-phase was more evident in the presence of zoledronate, particularly after the longest (72 h) period of exposure (additional data are given in Supplementary Table 1S). The results indicated that the NBPs down-modulated tumor cell proliferation via inducing cell cycle arrest at S-phase.

3.4. NBP treatment and cell invasion

The invasive behavior of cells cultured in the presence and absence of NBPs at different times was examined via trans-well migration assays. Here, 2×10^5 starved cells treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h were seeded into trans-well chambers with or without Matrigel™. The invasion percentage for each treatment was calculated by dividing the number of migrating cells in the Matrigel-coated chambers by the number of migrating cells in uncoated chambers (Supplementary Table 2S). Division of the invasion percentage for each treatment by the value for the corresponding control yields the invasion index. Pamidronate and zoledronate showed anti-invasive effects in BT20 cells at all experimental time points ($P < 0.001$). In contrast, risedronate had no effects on the invasive behavior of the BT20 cells at any time point. The anti-invasive effects of the NBPs on the T47D cells appeared after 48 h of treatment ($P < 0.001$). In the MDA-MB231 cells, a reduction of invasiveness was only observed after 72 h of pamidronate exposure ($P < 0.001$; Fig. 4 and Supplementary Table 2S). The results indicated that the anti-invasive effects of the NBPs were time dependent and cell type specific.

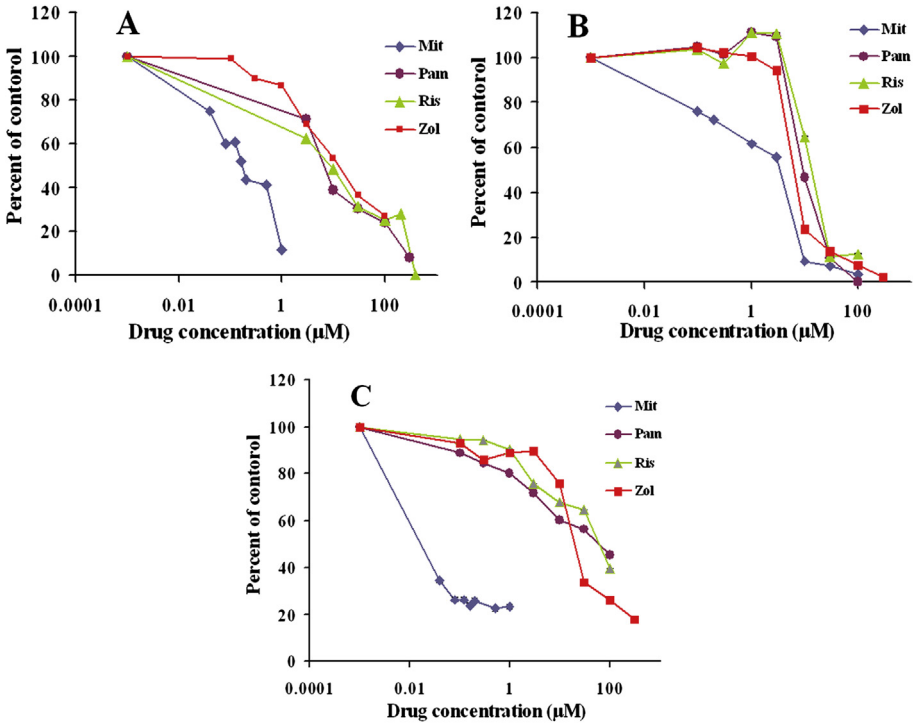

Fig. 1. Effects of mitoxantrone, pamidronate, risedronate, or zoledronate on the survival of BT20 (A), MDA-MB231 (B), and T47D (C) cells. The cells were cultured for 5 days with increasing doses of NBPs from 0 to 100 μM. Cell survival was measured by MTT assay. The values represent the means of three independent experiments performed in triplicate (Mean ± SE). Mit: mitoxantrone, Pam: pamidronate, Ris: risedronate, and Zol: zoledronate.

Table 1
IC_{50} values of NBPs and mitoxantrone for breast cancer cell lines.

Cells	Pamidronate IC_{50}[a] ± SE	Risedronate IC_{50} ± SE	Zoledronate IC_{50} ± SE	Mitoxantrone IC_{50} ± SE
T47D	69.4 ± 3.26	70.8 ± 4.13	22.2 ± 2.93	0.03 ± 0.004
MDA-	MB231 3.0 ± 0.21	9.5 ± 0.73	15.5 ± 1.02	7.3 ± 0.61
BT20	7.6 ± 0.43	9.0 ± 0.56	13.9 ± 0.98	0.169 ± 0.005

Data represent the mean ± standard error of three individual experiments.
[a] Drug concentration (μM) required for 50% inhibition of cell growth after 5 days of drug exposure.

4. Discussion and conclusion

Since the discovery of bisphosphonates in 1968 [19], these molecules have been clinically approved to reduce bone turnover, increase bone mass, confer significant fracture protection and eliminate bone metastasis due to common cancers, such as prostate and breast cancers. NBPs have also recently been the subject of many anticancer studies. These promising studies have provided valuable information about the pharmacological effects of NBPs. Despite the tremendous amount of work already conducted on this topic, several fundamental issues remain to be addressed, including the mechanisms underlying the promotion of apoptosis and cell cycle arrest, induction of T-cell cytotoxicity against tumor cells, inhibition of angiogenesis, inhibition of cell proliferation, reduction of cellular adhesion, and reduction of invasion by NBPs [4,20]. The established inhibition of protein prenylation in the mevalonate pathway does not allow interpretation of many of the new pharmacological effects of NBPs. Therefore, recent studies have aimed to identify new targets for these effects. For example, it has been reported that a DBF4 analog in a mutant yeast collection may be a

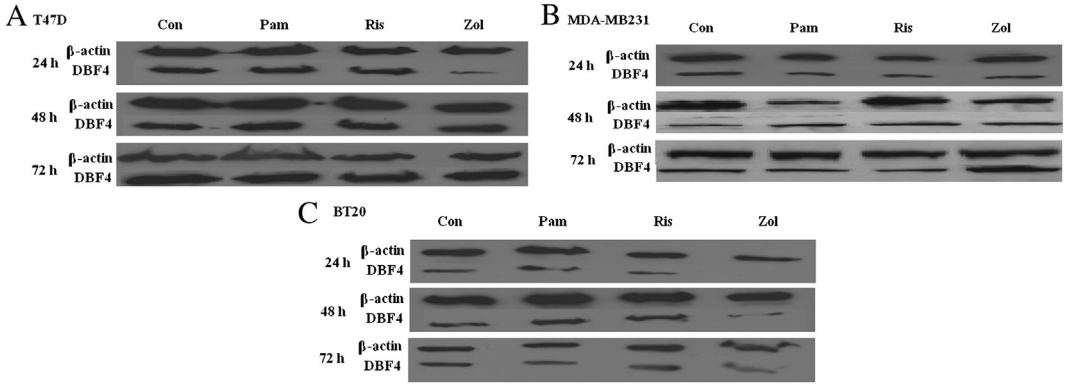

Fig. 2. Western blotting of DBF4 and β-actin. BT20, MDA-MB231, and T47D cells were treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h, and whole-cell lysates were prepared at each individual time point and subjected to western blotting to identify the levels of DBF4 expression. The blots were treated with antibodies against DBF4 and β-actin individually after stripping. β-Actin was used as an internal control. Pam: pamidronate, Ris: risedronate, and Zol: zoledronate.

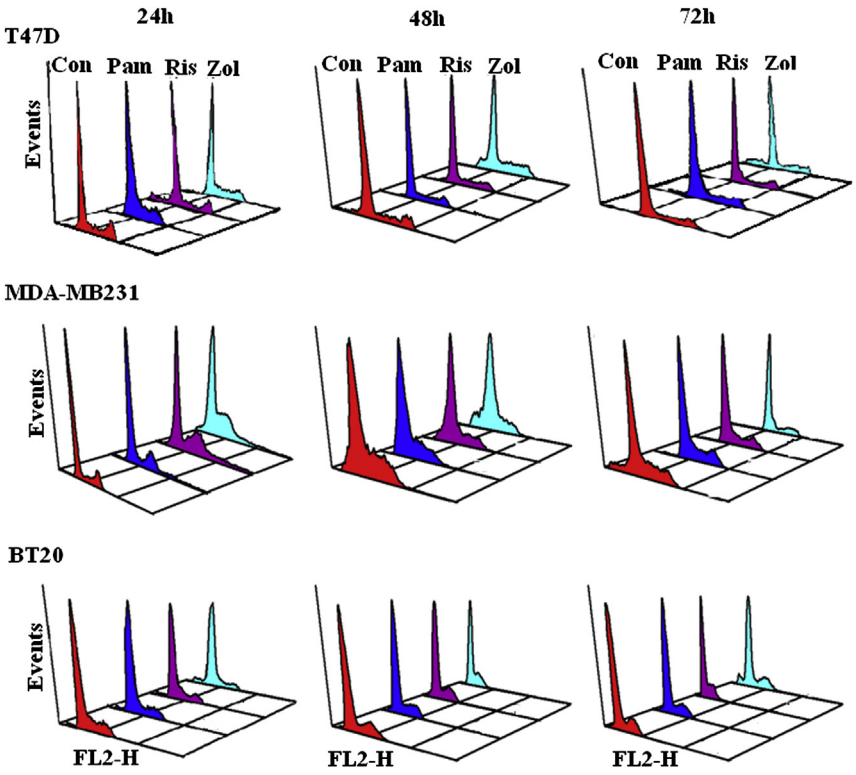

Fig. 3. Cell cycle phase alterations following NBPs treatment of human breast cancer cell lines. BT20, MDA-MB231, and T47D cells were treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h. The cells were then harvested, fixed and stained with propidium iodide for flow cytometric cell cycle analysis. Con: Control, Pam: pamidronate, Ris: risedronate, and Zol: zoledronate.

target of NBPs [8]. DBF4 is a highly conserved protein and represents an essential checkpoint at the G1/S transition for replication initiation and DNA synthesis via phosphorylation of the MCM helicase complex. The MCM helicase is assembled in replication forks during late G1-phase and triggers origin unwinding and firing of DNA synthesis. These results suggest that the execution of MCM function is dependent on DBF4 [21]. On the other hand, a DBF4-mediated phosphorylation cascade involving integrin and

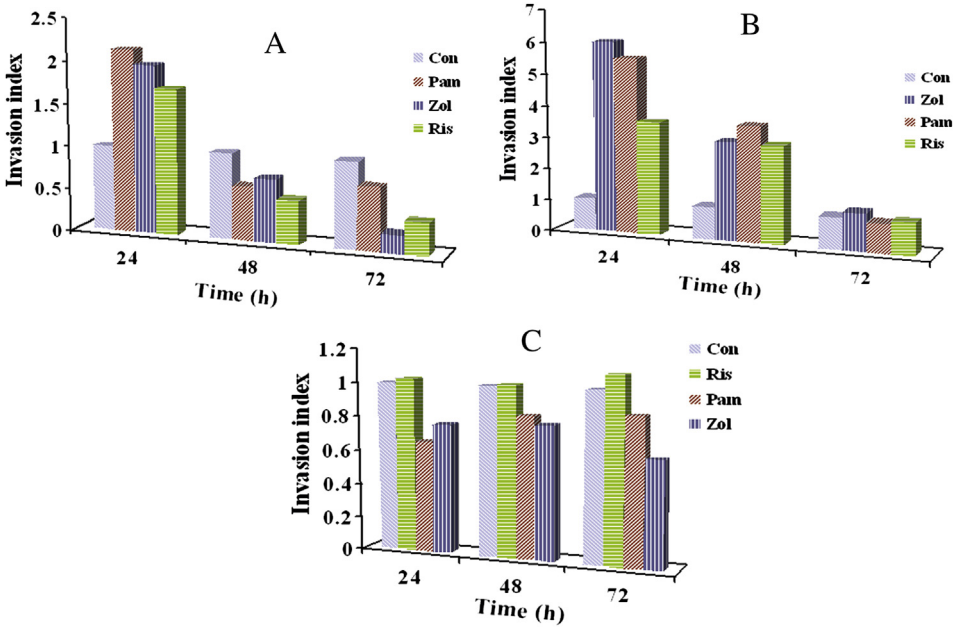

Fig. 4. The invasion index of the BT20 (A), MDA-MB231 (B), and T47D (C) cells in the presence of the NBPs. The cells were treated with 100 μM pamidronate, risedronate, or zoledronate for 24, 48, or 72 h. Invasion assays were performed using trans-well chambers coated with the basement membrane Matrigel. The migrating cells were counted in the absence or presence of Matrigel. The invasion index was quantified by division of the percentage of invasion for each treatment by the corresponding control values. The presented invasion data represent mean values from three separate experiments (Mean ± SE). Con: Control, Pam: pamidronate, Ris: risedronate, and Zol: zoledronate.

collagen was identified as playing a critical role in cell adhesion and migration. Bone, which is comprised of more than 90% collagen-I, is the most frequent site of metastasis for several common cancers. DBF4 depletion reduces integrin and collagen phosphorylation and cell attachment to collagen-I-coated surfaces and inhibits cell migration and invasion. Therefore, blocking this key molecule inhibits tumor cell migration and adhesion to the new sites, which could ultimately reduce metastasis [11]. Striking evidence has shown that the DBF4 protein is scarce or undetectable in normal tissues and cell lines but is up-regulated in approximately ~50% of human tumor cell lines, and some tumor cells also exhibit extra copies of the DBF4 gene. Therefore, increased DBF4 abundance may be a sign of malignancy in humans [22]. To gain a better understanding of whether the cell cycle arrest or invasion inhibition brought about by NBPs may occur via DBF4-mediated kinase activity, we have conducted extensive molecular analyses in several human cancer cell lines.

The present study showed that NBPs reduce overall cell growth in the BT20, MDA-MB231, and T47D breast cancer cell lines. These toxic effects are drug and cell specific (Table 1 and Fig. 1). Zoledronate was the most toxic agent in each of the three cell lines, and most of the cells tolerated risedronate better than the other NBPs. It also appears that MDA-MB231 cells are more sensitive to the effects of the NBPs than the other cell lines. As seen in the western blot analyses, zoledronate was the only NBP that caused a transient down-regulation of DBF4. However, all three NBPs caused cells to accumulate in S-phase of the cell cycle, with a corresponding decrease in the number of cells in other phases being observed during the experiment. The elevation of the percentage of cells in S-phase is not DBF4-mediated, at least based on the following two reasons. First, DBF4 down-regulation is expected to arrest cells in G1-phase; however, the transient down-regulation observed in zoledronate-treated cells was associated with accumulation of the cells in S-phase. Second, risedronate and pamidronate did not alter DBF4 expression, but the cell count in S-phase increased each day for all treatments during the three days of the experiment. The same simple reasons also refute the idea that the anti-invasive function of NBPs is DBF4 mediated. Interestingly, while DBF4 depletion is expected to reduce cell attachment and cell invasion, in the present study, though cell invasion was reduced after three days of NBP treatment, the DBF4 contents in the treated cells were almost equal to those in the controls at this time point. In general, the results obtained via western blotting and from cell cycle and invasion analyses in the investigated cancer cell lines provided insight into the DBF4-independent invasion inhibition and cell cycle arrest stimulated by NBP treatment, contributing to our understanding of the complexity of this regulatory mechanism [23]. Interestingly, although DBF4 expression was transiently down-regulated on the first day of zoledronate treatment in the three cell lines, we found no clear evidence that DBF4 down-regulation was associated with the effects of bisphosphonates treatment regarding cell cycle arrest or cancer cell invasion and metastasis. Therefore, novel signaling mediators should reveal potential targets for pharmacological interventions using NBPs to induce cell cycle arrest or prevent metastasis.

Conflict of interest

We certify that there is no conflict of interest with any financial organization.

Acknowledgments

The authors are grateful for financial support from Zanjan University of Medical Sciences (grant No. 19/3-3/3104). We also thank Dr. Ghamghami (Zanjan University of Medical Sciences) and Dr. Kalalinia (Bu-Ali research center, Mashhad University of Medical Sciences, Iran) for their valuable help.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2013.11.010

References

[1] N.B. Watts, D.L. Diab, Long-term use of bisphosphonates in osteoporosis, Clin. Endocrinol. Metab 4 (2010) 1555–1565.
[2] A. Tripathi, S. Pandey, S.V. Singh, N.K. Sharma, R. Singh, Bisphosphonate therapy for skeletal malignancies and metastases: impact on jaw bones and prosthodontic concerns, Prosthodontics (2010) 601–603.
[3] M.R. Smith, Antitumor activity of bisphosphonates, Clin. Cancer Res. 9 (2003) 5433–5434.
[4] D. Santini, S. Galluzzo, B. Vincenzi, G. Schiavon, E. Fratto, F. Pantano, G. Tonini, New developments of aminobisphosphonates: the double face of Janus, Ann. Oncol. 18 (2007) 64–67.
[5] S.G. Senaratne, G. Pirianov, J.L. Mansi, T.R. Arnett, K.W. Colston, Bisphosphonates induce apoptosis in human breast cancer cell lines, Br. J. Cancer 82 (2000) 1459–1468.
[6] P.G. Fournier, V. Stresing, F.H. Ebetino, P. Clezardin, How do bisphosphonates inhibit bone metastasis *in vivo*? Neoplasia 12 (2010) 571–578.
[7] F.P. Coxon, K. Thompson, M.J. Rogers, Recent advances in understanding the mechanism of action of bisphosphonates, Curr. Opin. Pharmacol. 6 (2006) 307–312.
[8] N. Bivi, M. Romanello, R. Harrison, I. Clarke, D.C. Hoyle, L. Moro, F. Ortolani, A. Bonetti, F. Quadrifoglio, G. Tell, D. Delneri, Identification of secondary targets of N-containing bisphosphonates in mammalian cells via parallel competition analysis of the barcoded yeast deletion collection, Genome Biol. 10 (2009) R93.
[9] M. Balestri, F. Taddei, M. Viceconti, M. Manfrini, Tibial bone adaptation after fibula osteotomy: an *in-vivo* analysis, in: Forum in Bone and Mineral Research 6th Meeting Napoli, 85, 2009, pp. 451–462.
[10] B.Q. Guo, J. Romero, B.J. Kim, H. Lee, High levels of Cdc7 and Dbf4 proteins can arrest cell-cycle progression, Eur. J. Cell Biol. 84 (2005) 927–938.
[11] Y. Chen, B. Lu, Q. Yang, C. Fearns, J.R. Yates, J.D. Lee, Combined integrin phosphoproteomic analyses and siRNAbased functional screening identified key regulators for cancer cell adhesion and migration, Cancer Res. 69 (2009) 3713–3720.
[12] F. Elahian, F. Kalalinia, J. Behravan, Dexamethasone downregulates BCRP mRNA and protein expression in breast cancer cell lines, Oncol. Res. 18 (2009) 9–15.
[13] F. Elahian, F. Kalalinia, J. Behravan, Evaluation of indomethacin and dexamethasone effects on BCRP-mediated drug resistance in MCF-7 parental and resistant cell lines, Drug Chem. Toxicol 33 (2010) 113–119.
[14] F. Elahian, B. Moghimi, F. Dinmohammadi, M. Ghamghami, M. Hamidi, S.A. Mirzaei, The anticancer agent prodigiosin is not a multidrug resistance protein substrate, DNA Cell. Biol. 32 (2013) 90–97.
[15] S.A. Mirzaei, M.T. Yazdi, Z. Sepehrizadeh, Secretory expression and purification of a soluble NADH cytochrome b5 reductase enzyme from *Mucor racemosus* in *Pichia pastoris* based on codon usage adaptation, Biotechnol. Lett. 32 (2010) 1705–1711.
[16] H. Lage, C. Perlitz, R. Abele, R. Tampé, M. Dietel, D. Schadendorf, P. Sinha, Enhanced expression of human ABC-transporter tap is associated with cellular resistance to mitoxantrone, FEBS Lett. 503 (2001) 179–184.
[17] K. Velpula, V. Dasari, A. Tsung, C. Gondi, J. Klopfenstein, S. Mohanam, J. Rao, Regulation of glioblastoma progression by cord blood stem cells is mediated by downregulation of cyclin D1, PLoS ONE 6 (2011) 1–13.
[18] B. Grismayer, S. Sölch, B. Seubert, T. Kirchner, S. Schäfer, G. Baretton, M. Schmitt, T. Luther, A. Krüger, M. Kotzsch, V. Magdolen, Rab31 expression levels modulate tumor-relevant characteristics of breast cancer cells, Mol. Cancer 11 (2012) 1–14.
[19] H. Fleisch, R. Russell, S. Bisaz, P. Casey, R. Mühlbauer, The influence of pyrophosphate analogues (diphosphonates) on the precipitation and dissolution of calcium phosphate in vitro and *in vivo*, Calcif. Tissue Res. 2 (1968), 10–10a.
[20] R. Verdijk, H.R. Franke, F. Wolbers, I. Vermes, Differential effects of bisphosphonates on breast cancer cell lines, Cancer Lett. 246 (2007) 308–312.
[21] Y.J. Sheu, B. Stillman, Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression, Mol. Cell 24 (2006) 101–113.
[22] D. Bonte, C. Lindvall, H.Y. Liu, K. Dykema, K. Furge, M. Weinreich, Cdc7-Dbf4 kinase overexpression in multiple cancers and tumor cell lines is correlated with p53 inactivation, Neoplasia 10 (2008) 920–931.
[23] F. Elahian, Z. Sepehrizadeh, B. Moghimi, S.A. Mirzaei, Human cytochrome b5 reductase: structure, function, and potential applications, Crit. Rev. Biotechnol (2012), http://dx.doi.org/10.3109/07388551.2012.732031 (Epub ahead of print).